# Immunocytochemical and enzymatic detection of lysozyme in human colon carcinoma cell lines[1]

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*Summary.* Six of a total of 14 human colon carcinoma cell lines produce and secrete lysozyme in vitro. Three also produce the enzyme when propagated in vivo in athymic mice. None of the lysozyme positive cells stained in a manner typical of Paneth cells. Additionally, lysozymes from all six colon lines possess identical molecular weights ( ~ 14,000 daltons).
*Key words.* Human colon carcinoma cells; lysozyme; immunocytochemistry.

Lysozyme has been detected immunohistochemically and enzymatically in several normal human secretions and tissues including saliva, tears, milk, serum, placenta, stomach and small intestine (see Fett et al.[5] and references cited therein for review). In the majority of those cases lysozyme synthesis occurs in leukocytes, tissue macrophages, or secretory epithelium, including Paneth cells. Malignant transformation of these cell types might be expected to increase overall lysozyme production as has been observed in Hodgkin's disease[6], various forms of leukemia[7], and recently in solid tumors of the upper gastrointestinal tract[8–11] attributable in some cases to the presence of neoplastic Paneth cells[10–12]. While gastric carcinomata have been reported to produce lysozyme quite frequently, tumors of the lower gastrointestinal tract have not[8–10].

Our finding that lysozyme is a major protein secreted in vitro by an established human colon adenocarcinoma cell line, HT-29[5], led to the present study which shows that lysozyme is produced in six of fourteen human colon carcinoma cell lines examined. Moreover, lysozyme-producing cells maintain their capacity to synthesize lysozyme when propagated in vivo in athymic mice and do not resemble typical Paneth cells.

*Materials and methods. Cell lines and culture conditions.* All cells were of human origin and included fourteen colon carcinoma cell lines, a lung carcinoma line, a fibrosarcoma line, and a normal colon fibroblastic cell line (table). All except the HT-29 cell line[13] were obtained from the American Type Culture Collection, Rockville, MD. Cells were grown routinely as monolayer cultures utilizing Dulbecco's modified Eagle's medium (DME) supplemented with glucose (4.5 mg/ml), gentamicin (50 mg/ml), fungizone (0.5 mg/ml), L-glutamine (2 mM), and 5% fetal bovine serum (DME/5). Cultures were incubated at 37 °C in a humidified, 7% $CO_2$ atmosphere in air. Medium was changed every 2–3 days, and cells were subcultured and harvested using standard trypsinization techniques.

*Preparation of antibodies.* Commercially available rabbit anti-human lysozyme immunoglobulin (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) was dialyzed and lyophilized prior to use. A control immunoglobulin fraction was prepared from the serum of a non-immune New Zealand White rabbit by standard ammonium sulfate precipitation followed by protein-A Sepharose and diethylaminoethyl cellulose chromatography. Anti-human lysozyme activity was absorbed from the commercial immunoglobulin preparation utilizing an affinity column prepared by coupling human placental(Pl-) lysozyme (Alpha Therapeutic Corp., Los Angeles, CA) to Affi-gel 10.

*Enzyme assays.* Cells from the seventeen lines listed above were plated in triplicate into 25-cm$^2$ Falcon T-flasks at $1 \times 10^6$ viable cells in 4 ml of DME/5. After seven days in culture conditioned medium was harvested, filtered, and frozen at −20 °C for one week prior to assay. Lysozyme activity in supernatant fluids was determined spectrophotometrically according to the turbidometric method of Locquet et al.[14] as described[5].

*Histology and cytology.* For the study of tumor cells in vitro, cultured cells were harvested during mid-log growth by trypsinization, washed twice in DME, resuspended at $5 \times 10^5$ cells/ml in DME plus 1% bovine serum albumin, and centrifuged onto microscope slides by means of a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA). Preparations were fixed for 5 min with 10% phosphate buffered formalin immediately prior to staining.

For the study of tumor cells in vivo, tumors were grown in athymic (nude) Balb/c mice (Charles River Laboratories, Wilmington, MA) by the s.c. injection of $1 \times 10^6$ tumor cells harvested as above into the shoulder area. When tumors measured > 5 mm in diameter, they were removed, fixed at 4 °C for 2 h in 10% phosphate buffered formalin, and then overnight in 70% ethanol. Specimens were then dehydrated through graded alcohols and xylene and embedded in paraffin.

Cytocentrifuge, formalin-fixed preparations of tumor cells and deparaffinized tumor sections cut at 4 mm were stained with hematoxylin and eosin and with phloxine-tartrazine for Paneth cell granules[15].

For localization of lysozyme the immunoperoxidase bridge technique was used, in conjunction with the rabbit anti-lysozyme antibody, as described previously[16]. Control immunoglobulin reagents included non-immune rabbit immunoglobulin and rabbit anti-lysozyme absorbed with Pl-lysozyme.

*Processing of conditioned medium.* The six colon lines found to produce lysozyme were grown as monolayer cultures in 75-cm$^2$ Falcon T-flasks in DME/5 as above. Conditioned medium was harvested at confluence, filtered, dialyzed against distilled water, and lyophilized. Each lyophilized preparation ( ~ 200 mg) was dissolved in 2 ml of 100 mM sodium phosphate, pH 6.6 (running buffer) and applied to a 1.5 × 8.0 cm column of carboxymethyl (CM)-cellulose (Whatman, grade CM 52). The column was

Lysozyme production by human established cell lines propagated in vitro and in vivo

| Cell lines | | Lysozyme production | | |
|---|---|---|---|---|
| | | In vitro | | In vivo[c] |
| Designation | Type | Extracellular[a] µg/ml | Cellular[b] | |
| HT-29 | colon adenocarcinoma | 1.20 | 3+ | 3+ |
| WiDr | colon adenocarcinoma | 1.15 | 2+ | 1+ |
| COLO 201 | colon adenocarcinoma | 0.20 | 2+ | —[e] |
| COLO 205 | colon adenocarcinoma | 0.20 | 1+ | 1+ |
| COLO 320 DM | colon adenocarcinoma | ND[d] | 0 | — |
| SW 403 | colon adenocarcinoma | 0.10 | 1+ | — |
| SW 480 | colon adenocarcinoma | ND | 0 | 0 |
| SW 620 | colon adenocarcinoma[f] | ND | 0 | — |
| SW 837 | colon adenocarcinoma | ND | 0 | — |
| SW 948 | colon adenocarcinoma | ND | 0 | — |
| SW1116 | colon adenocarcinoma | 0.20 | 2+ | — |
| SW 1417 | colon adenocarcinoma | ND | 0 | — |
| LoVo | colon adenocarcinoma[f] | ND | 0 | — |
| HCT-15 | colon adenocarcinoma | ND | 0 | — |
| A549 | lung carcinoma | ND | 0 | 0 |
| HT-1080 | fibrosarcoma | ND | 0 | — |
| CCD-18Co | fibroblast (normal colon) | ND | 0 | — |

[a] Extracellular lysozyme determined enzymatically. Sensitivity of assay is ≥ 25 ng/ml. [b] Cytocentrifuge preparations stained for lysozyme with the immunoperoxidase bridge technique. Reactivity was graded on a semi-quantitative scale from 0 to 3+ based on staining intensity. [c] Tissue sections stained and graded as for cytocentrifuge preparations. [d] ND; not detectable. [e] —; not determined. [f] These lines were established from metastatic nodules of patients with primary colon adenocarcinoma.

washed with running buffer and bound, lysozyme-containing material was then eluted with running buffer containing 1 M NaCl. This fraction (designated CM 2) was dialyzed against water, lyophilized, and analyzed by SDS-PAGE, electrophoretic transfer, and immunostaining as described below.

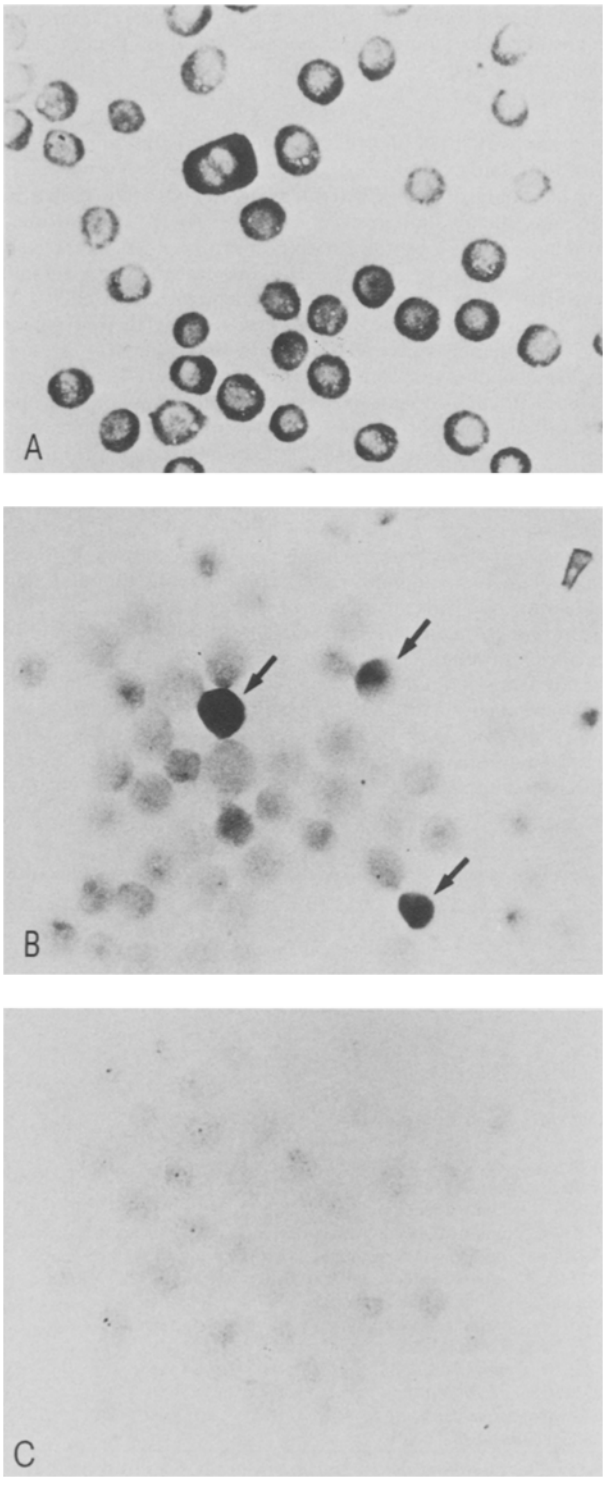


Figure 1. Immunostaining of cultured cells with anti-lysozyme using the immunoperoxidase technique. *A* HT-29 colon carcinoma (600 × ); *B* COLO 205 colon carcinoma. Note heterogeneous staining pattern compared to HT-29 cells *(A)*. Arrows identify some of the most intensely reactive cells (600 × ); *C* HT-29 cells stained with anti-lysozyme following absorption with purified human Pl-lysozyme (600 × ).

*Gel electrophoresis procedures.* SDS-PAGE was performed in the absence of reducing agents utilizing 15% gels with a 5% stacking gel according to Laemmli[17]. Electrophoretic transfer to nitrocellulose was performed according to the method of Towbin et al.[18]. Following transfer the sheets were blocked[19], incubated with rabbit anti-human lysozyme or rabbit non-immune immunoglobulin (40 µg/ml), and washed. The sheets were then incubated with alkaline phosphatase-conjugated goat anti-rabbit antiserum (Boehringer-Mannheim, Indianapolis, IN) diluted 1:250, washed, and developed with nitro blue tetrazolium/indoxyl phosphate according to Blake et al.[20].

*Results.* Lysozyme activity was detected enzymatically in the conditioned medium of six of the seventeen cell lines examined (table). All six were derived from colon carcinomata and included HT-29, WiDr, COLO 201, COLO 205, SW 403, and SW 1116. The extent of extracellular activities varied over a circa 5–10-fold range with the HT-29 and WiDr cell lines producing the highest level of 1.20 and 1.15 µg of lysozyme per ml of medium, respectively.

Among the seventeen cell lines tested for intracellular lysozyme production by immunoperoxidase staining, six showed varying degrees of positive reactivity correlating precisely with those shown to have lysozyme in their conditioned medium (table). Of these, HT-29 stained most intensely in direct agreement with the level of lysozyme activity found extracellularly. Although virtually all HT-29 cells immunostained to some degree, reactivity of a minor population (1-8% of total) of large-sized multinuclear cells was most intense (fig. 1a). However, in all positive cells the intracellular distribution of lysozyme appeared to be predominantly cytoplasmic. The staining patterns of WiDr, COLO 201, SW 403, and SW 1116 were similar to that of HT-29, although compared to HT-29 the overall reactivity was somewhat weaker. Most cells in each population were positive, with the stongest reactivity seen in large multinuclear cells. In contrast, the staining pattern of COLO 205 cells was more heterogeneous consisting of a few strongly positive cells and others which were completely negative (fig. 1b).

The substitution of non-immune rabbit immunoglobulin or absorbed rabbit anti-lysozyme for the first step in the bridge sequence eliminated all staining in each instance as illustrated in figure 1c for HT-29. Neither human lung carcinoma (A 549), fibrosarcoma (HT 1080), nor normal colon fibroblasts (CCD-18Co) exhibited lysozyme immunoreactivity.

None of the six colon tumor cell lines which produce lysozyme morphologically resemble Paneth cells after staining with hematoxylin and eosin, nor did any of the cell lines display typical Paneth cell granules after staining with phloxine-tartrazine. In addition, cells from HT-29, WiDR, and COLO 205 did not display Paneth cell granules after growth in athymic mice (see below).

To determine whether lysozyme production was limited to cells cultured in vitro or was also associated with tumors propagated in vivo, the three cell lines producing the most lysozyme in vitro, HT-29, COLO 205, and WiDr, were grown in athymic mice and then examined for lysozyme immunoreactivity. All three retained this property in vivo. Additionally, SW 480 (colon carcinoma) and A 549 (lung carcinoma), both negative for lysozyme in vitro, also remained negative in vivo (table). A lysozyme-stained, paraffin embedded section of the HT-29 tumor is shown in figure 2.

Conditioned medium was harvested from each of the six colon carcinoma lines producing lysozyme (table) and fractionated on CM-cellulose. The lysozyme-containing CM 2 fractions were subjected to SDS-PAGE followed by electrophoretic transfer and immunostaining. The staining patterns demonstrated the specificity of the immune reaction and the molecular weight identity ( ~ 14,000) of each of the six lysozymes. Substitution of non-immune rabbit immunoglobulin for the specific anti-lysozyme reagent abolished this staining.

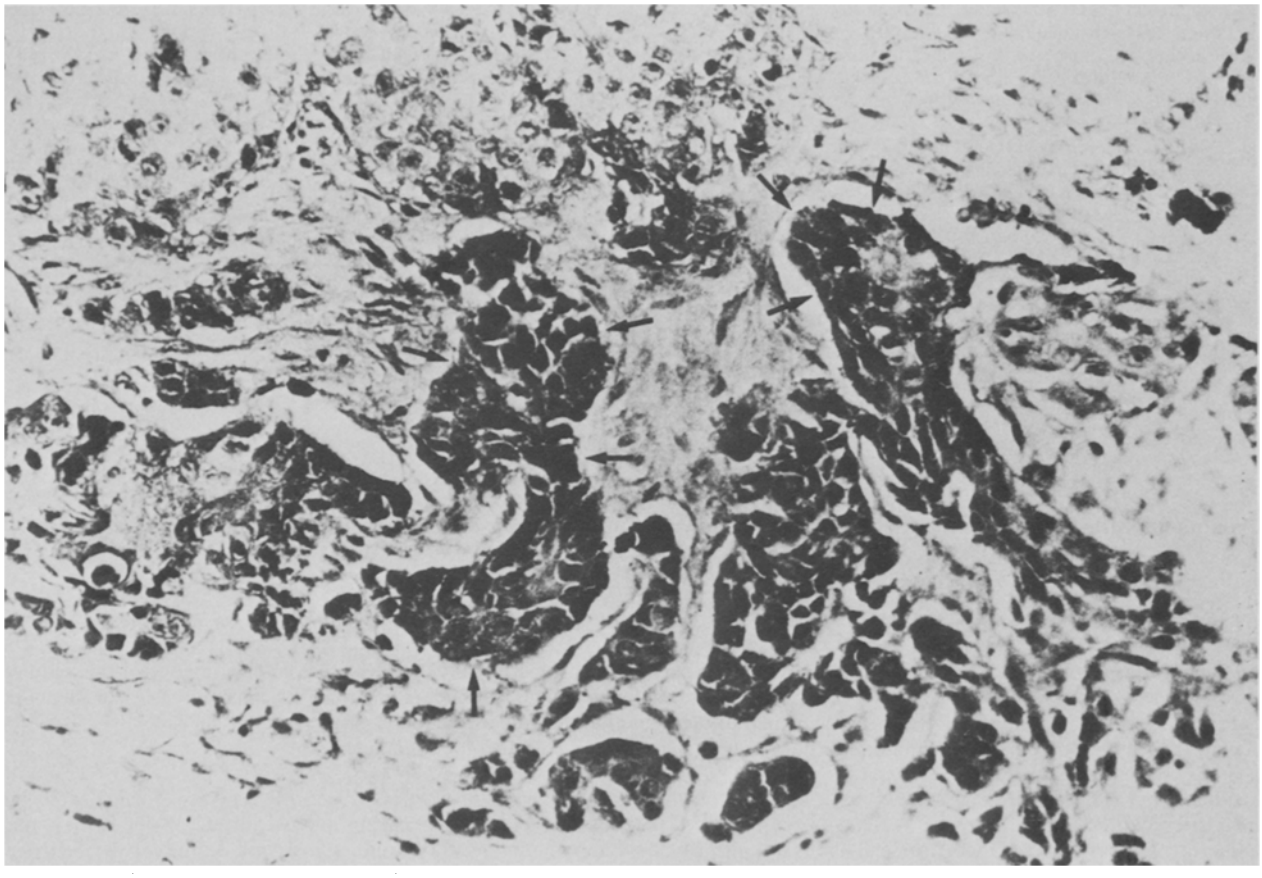

Figure 2. HT-29 tumor from an athymic mouse stained with anti-lysozyme. Neoplastic portions of the tumor are strongly positive (arrows), whereas little reactivity is detected in fibrous stroma (300 × ).

*Discussion.* The presence of lysozyme in neoplastic cells of myelocytic and monocytic leukemias and in certain malignancies of the gastrointestinal tract is well documented, and in these situations its occurrence may have important diagnostic and prognostic significance[7,21]. Although gastric tumors frequently produce lysozyme[8-11], those of the colon have not been found to do so thus far. In two studies performed to date only a few cells in one of a total of forty-two carcinomata of the colon screened systematically produced lysozyme[9,10]. This could relate in part to the observation that epithelial cells which are normally lysozyme-positive are not present in the large intestine[22,23]. However, neoplasms containing Paneth cells have been found in the colon, albeit very rarely[24-28], though it is not known whether or not they then continue to produce lysozyme in all instances.

In the present study, six of the fourteen colon adenocarcinoma lines that were examined produced and secreted lysozyme, as judged by both immunocytologic and enzymatic criteria. Furthermore, morphologically none of these cell lines resembled Paneth cells, usually found in the stomach and small intestine, nor were Paneth cell granules demonstrable by special staining techniques. Additionally, three of the lysozyme-producing cell lines when implanted into nude mice continued to synthesize this protein. Thus, the capacity to produce lysozyme is not limited solely to tumor cells grown in vitro. At present, however, it is not known why some, but not other, adenocarcinomas of the colon produce this enzyme nor what effects such synthesis might have on in vitro or in vivo growth.

The observation that several colon carcinoma cell lines synthesize lysozyme contrasts with studies of lysozyme production by solid tumors of the colon[9,10]. The reasons for this discrepancy are unknown, although additional examination of solid tumors may conceivably reveal that lysozyme-producing malignancies of the colon are more frequent than observed heretofore.

Clearly, the question remains as to what role(s), if any, lysozyme plays in the pathogenesis of colon carcinoma or what potential utility it might serve as a tumor marker for the detection, diagnosis, or monitoring of disease. Pertinent to the above, we have previously detailed potential functions of lysozyme, other than its classical role as an antibacterial or antiviral agent, which may be relevant to the malignant process[5]. In light of the current investigations, further studies appear warranted to resolve the issues posed above and efforts are currently underway to examine the relevance of this enzyme in tumor growth and cell metabolism.

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## Immunoquantitation of some cytochrome P-450 isozymes in liver microsomes from streptozotocin-diabetic rats

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*Summary.* Streptozotocin-diabetes in rats leads to a decrease of cytochrome P-450 UT-A (the major form in control rats) and an increase of cytochrome P-450 PB-B (the major one induced by phenobarbital treatment) in liver microsomes. The increased benzphetamine-N-demethylase activity can be related to the induction of cytochrome P-450 PB-B.
*Key words.* Cytochrome P-450; immunoquantitation; liver microsomes; streptozotocin-diabetes.

Modifications of monooxygenase activities in liver microsomes from streptozotocin (STZ)-diabetic rats have been described in numerous reports[1-4]. Since cytochrome P-450 is a multiprotein family[5-6], these modifications of enzyme activities in STZ-diabetic rats result from changes in the cytochrome P-450 isozymic pattern. The purpose of this study was to investigate this pattern by means of immunoquantitation of some specific isozymes of cytochrome P-450, and to try to correlate it with the monooxygenase activities exhibited by diabetic rats. This new approach may give further insight into the molecular mechanism which sustains drug metabolism in diabetic rats.

*Materials and methods.* Male rats of the Sprague-Dawley strain (IFACREDO, France), 2½–3 months old were used. Diabetes was produced by a single intrafemoral injection of STZ (80 mg/kg, freshly dissolved in saline solution, pH 4.5) under light ether anesthesia. Glucosuric animals were killed two weeks later. Microsomal membranes were prepared, and cytochrome P-450 and monooxygenase activities were determined, as previously reported[7]. The major cytochromes P-450 isolated from untreated, phenobarbital (PB), B-naphtoflavone (BNF) or isosafrole (ISF)-treated rats were respectively called cytochrome P-450 UT-A, PB-B, BNF-B and ISF-G[8]. Antibodies were prepared as previously described[7]. Anti-UT-A and anti-PB-B were rendered monospecific as indicated[9]. The immunoquantitation of the cytochrome P-450 isozymes was performed by Western Blots as described by Guengerich et al[10].

*Results and discussion.* In addition to the increase of both cytochrome P-450 and aniline hydroxylase shown before[1-4], we report the increase of benzphetamine-N-demethylase activity in liver microsomes from STZ-diabetic rats (table 1).

The immunoquantitation technique reveals that diabetes does not affect either cyt.P-450 ISF-G or cyt. P-450 BNF-B but that the disease is associated with depressed levels of cyt. P-450 UT-A (by 85%) and increased levels of cyt. P-450 PB-B (8.5-fold) (table 2).

It has been reported that cyt. P-450 PB-B supports with great specificity the N-demethylation of benzphetamine[7]. Therefore it may be suggested that the increased benzphetamine-N-demethylase activity in microsomes from STZ-diabetic rats is the result of the increased level of cyt. P-450 PB-B. This is further indicated by the fact that identical amounts of anti-cyt. P-450 PB-B immunoglobulins produce quite similar inhibitions of the benzphetamine-N-demethylase activity in microsomes from either STZ or PB-treated rats[11].

Thus, the PB-treatment and the STZ-produced diabetes both affect the content of cyt. P-450 PB-B (which is increased) and of cyt. P-450 UT-A (which is decreased). However, they differ by the intensity of their effects: diabetes depresses the level of cyt. P-450 UT-A more than PB-treatment does, but it is less potent in increasing the level of cyt. P-450 PB-B as compared with results obtained in control and PB-treated rats by Guengerich et al.[8] and in our own laboratory (T. Cresteil et al., submitted for publication). Moreover, unlike PB, which acts directly on cyt. P-450 synthesis, the molecule of streptozotocin by itself is not involved in the modification of cytochrome P-450 content and monooxygenase activities: these modifications are the result of the modified hormonal state[3-12].

Moreover, the total immunoquantitated isozymes in liver microsomes from diabetic rats do not account for the level of cytochrome P-450 assayed by the spectrophotometric method. This means that diabetes promotes the synthesis of specific isozymes which differ from the main ones produced by classical inducers. A cytochrome P-450 with high activity towards aniline has been isolated from alloxan-diabetic rats by Past and Cook[13]; it might be one of these specific cytochromes P-450.

Table 1. Cytochrome P-450 content and monooxygenase activities in liver microsomes from normal and diabetic rats

| | *Cytochrome P-450 | Aniline hydroxylase | Benzphetamine N-demethylase |
|---|---|---|---|
| Control rats | 0.87 ± 0.03 | 0.53 ± 0.05 | 2.70 ± 0.63 |
| Diabetic rats | 1.20 ± 0.04 | 1.19 ± 0.08 | 7.96 ± 1.23 |

*expressed as nmol/mg of microsomal proteins. Enzyme activities are expressed as nmol of product formed × $min^{-1}$ × $nmol^{-1}$ cyt. P-450. Results: mean ± SE of 4–7 animals.